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(54) Evaluating an immunoassay test sheet by immunoassay

(57) The test sheet comprises a fibrous sheet carrying either calcium phosphate (hydroxyapatite) particles or alternatively polymer particles coated with calcium phosphate. An antigen or antibody is immobilized on the calcium phosphate. The resulting test sheet is evaluated by specifically binding a further reactive antibody or antigen to said immobilised antigen or antibody followed by detection of said reactive antibody or antigen by a labelled compound which produces a colour change. The level of colour is used to quantitatively evaluate the test sheet.

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METHOD OF EVALUATING A TESTING ELEMENT FOR ANTIGENS OR ANTIBODIES

The present invention relates to a method of evaluating a testing element for antigens or antibodies, more particularly, the present invention relates to a method of evaluating functions or characteristics of the testing element which contains a known antigen or antibody immobilized thereon and is used in a diagnosis of a variety of infectious diseases based on an antigen-antibody reaction.

Various types of testing elements such as testing beads, testing sheets and others which comprise an immobilized antigen or antibody of the known type have been provided for use in detecting an antigen or antibody in a biological fluid such as saliva, blood, lymph, excreta and other fluids. Many of the well-known testing elements contain an antigen or antibody immobilized thereon by an adsorption action of the carrier used. However, in these testing elements, it is difficult to always obtain a constant adhesion and thus immobilization of the antigen or antibody to the elements. When the amount of the

immobilized antigen or antibody varies, it becomes necessary to evaluate the functions of the testing element.

An object of the present invention is to provide an evaluation method for testing elements with an immobilized antigen or antibody, which can easily determine a quantity of the immobilized antigen or antibody and also can stably evaluate the functions of the testing elements.

According to the present invention there is provided a method of evaluating a testing element having an immobilized antigen or antibody on an immobilizing carrier thereof, which comprises the steps of:

contacting the testing element with a solution containing an antibody or antigen reactive with said immobilized antigen or antibody,

specifically bonding a labelling compound to said reactive antibody or antigen to thereby cause a color-developing reaction of said labelling compound, and

determining an amount of the antigen or antibody immobilized on said testing element as a function of the level of the produced color.

Using the evaluation method of the present invention, it becomes possible to easily carry out a quantitative determination of the antigen or antibody immobilized on a

carrier of the testing element with reference to the color-developing reaction of the labelling compound used, and also stably evaluate the functions and characteristics of the testing element for the antigens or antibodies.

Examples of the present invention will now be described.

As mentioned above, a testing element is used having an immobilized antigen or antibody on an immobilizing carrier. The testing element may be formed from a wide variety of immobilizing carriers with an antigen or antibody fixed to the selected immobilizing carrier. Typical examples of a suitable testing element, although they are not restricted to the below mentioned, include:-

1. The testing element in the form of detection beads comprising particles of a calcium phosphate compound having a Ca/P ratio of about 1.0 to 2.0 and an average particle diameter of about 1 to 10000 microns, as an immobilizing carrier, having immobilized thereon an antigen or antibody.

2. The testing element in the form of detection sheets comprising a fibrous aggregate with carried particles of a calcium phosphate compound, as an immobilizing carrier, having immobilized thereon an antigen or antibody. Preferably, the calcium phosphate compound

used herein has a Ca/P ratio of about 1.0 to 2.0 and an average particle diameter of about 1 to 10000 microns. The testing element is preferably in the form of detection sheets for antigens or antibodies. See, for example, Japanese Patent Application No. 6-214706.

3. The testing element in the form of detection beads comprising a granular composite of polymer comprising polymeric granules having coated on a surface thereof a calcium phosphate compound, at least a part of particles of said calcium phosphate compound being penetrated in said polymeric granules, as an immobilizing carrier, having immobilized thereon an antigen or antibody. Preferably, the calcium phosphate compound used herein has a Ca/P ratio of about 1.0 to 2.0. The granular composite of polymer used herein is disclosed in, for example, Japanese Unexamined Patent Publication (Kokai) No. 7-194970.

4. The testing element in the form of detection beads comprising a granular composite of polymer comprising polymeric granules having coated on a surface thereof a calcium phosphate compound, said polymeric granules of said granular composite being dyed, as an immobilizing carrier, having immobilized thereon an antigen or antibody. Preferably, the calcium phosphate compound used herein has a Ca/P ratio of about 1.0 to 2.0. This type of testing element is disclosed in, for example, Japanese Unexamined

Patent Publication (Kokai) No. 7-174762.

In the present testing element, when a calcium phosphate compound is used as an immobilizing medium for antigens or antibodies, the calcium phosphate compound used herein is not restrictive, and suitable calcium phosphate compounds include a wide variety of calcium phosphate compounds having a Ca/P ratio in the range of about 1.0 to 2.0. For example, one or more of $Ca_{10}(PO_4)_6(OH)_2$, $Ca_{10}(PO_4)_6F_2$, $Ca_{10}(PO_4)_6Cl_2$, $Ca_3(PO_4)_2$, $Ca_2P_2O_7$, $Ca(PO_3)_2$, $Ca_4O(PO_4)_2$, and $CaHPO_4$ may be used as the calcium phosphate compounds. Among these calcium phosphate compounds, the most preferred one is a calcium phosphate compound which contains hydroxyapatite as a principal component thereof. These calcium phosphate compounds may be produced in any conventional method including a wet process, a dry process and other processes.

The particles of the calcium phosphate compound used herein can be produced by using any conventional granulation method . For example, they can be produced by spray-drying a slurry of the calcium phosphate compound and then calcinating the dried product to obtain the intended particles of the calcium phosphate compound. Preferably, sieve and other separation means may be used to select the particles of the calcium phosphate compound having the predetermined range of the particle size depending on the

intended use of the particles.

The calcium phosphate compound has an excellent adsorption function to antigens or antibodies such as bacteria, virus and others. However, in order to ensure an immobilization of the antigens or antibodies thereon, it is preferred that after adsorption of the antigens or antibodies on the calcium phosphate compound, the adsorbed antigens or antibodies are further treated with a cross-linking agent such as glutaraldehyde and the like, a binding agent such as formaldehyde, silane coupling agent and the like, or osmium tetrachloride. Examples of suitable silane coupling agent include 3-glycidoxypropyl trimethoxysilane, 3-thiopropyl trimethoxysilane, 2-(3-trimethoxysilylpropyldithio)-5-nitropyridine, 3-aminopropyl triethoxysilane, 3-chloropropyl dimethoxymethylsilane and the like.

After immobilization, it is preferred that antigen- or antibody-unadsorbed sites of the immobilizing carrier are treated with a blocking agent. The blocking agent used herein is not restricted, insofar as it is able to be adsorbed on said unadsorbed sites of the calcium phosphate compound and does not adversely affect the subsequently caused antigen-antibody reaction. Suitable blocking agents include, for example, proteins such as casein and albumin.

The evaluation method of the testing element

of the present invention,

comprises the steps of:-

contacting the testing element with a solution containing an antibody or antigen reactive with said immobilized antigen or antibody;

specifically bonding a labelling compound to said reactive antibody or antigen to thereby cause a color developing reaction of said labelling compound; and

determining an amount of the antigen or antibody immobilized on said testing element as a function of the level of the produced color.

Preferably, the testing element is contacted with a solution containing a known amount of antibody or antigen reactive with said immobilized antigen or antibody.

In the practice of the present evaluation method, preferably, an antibody or antigen reactive with an immobilized antigen or antibody is separately prepared and then diluted with a solvent to prepare a series of the diluted solution of antibody or antigen with different dilution degree. Suitable solvents useful in the preparation of the diluted solution are, for example, physiological saline, phosphate buffer solution (PBS) and the like. Then, the resulting solution of antibody or antigen is contacted with a testing element containing an immobilized antigen or immobilized antibody to be evaluated, to thereby cause an

antigen-antibody reaction. The thus formed composite of antigen and antibody is then labelled with a labelling compound capable of specifically bonding to said composite and generating a color developing reaction of the labelling compound. The color developing reaction thus generated can be utilized as a measure of the quantitative determination of said immobilized antigen or immobilized antibody.

The evaluation of the testing element can be carried out as follows.

A reference testing element having a standard amount of an immobilized antigen or antibody is contacted with a series of diluted solutions of a known amount of antibody or antigen with different dilution degree to produce an antigen-antibody composite, and then with a labelling compound capable of generating a color developing reaction with said antigen-antibody composite. The so developed color is used as reference.

Separately, the testing element to be evaluated, to which an unknown amount of an antigen or antibody is immobilized, is contacted with the same series of diluted solutions of the antigen or antibody as above, and then with the same labelling compound as above. The density of the developed color is compared with the density of reference color.

When the testing element to be evaluated shows the same color density as that of reference testing element, both elements have the same amount of the immobilized antigen or antibody. Accordingly, the amount of the antigen or antibody immobilized to the testing element to be evaluated can be determined from the dilution degree of the used solution of the antibody or antigen when the same color density is developed.

The labelling compound used herein is not restrictive, insofar as it may specifically bond to the antibody or antigen which has reacted with the immobilized antigen or immobilized antibody. Suitable labelling compounds, although they are not restricted to the below-mentioned, include, antigens or antibodies which contain peroxidase, glucose oxidase, tyrosinase, acidic phosphatase or alkaline phosphatase as a labelling enzyme.

Using the above-listed labelling enzymes, it becomes easy to induce the subsequent color developing reaction and thus the quantitative determination for the amount of the antigen or antibody immobilized on the carrier. Namely, the color developing reaction can be easily induced by using a substrate which can be labelled with an enzyme and can develop a color upon reaction with said enzyme.

The substrate advantageously used herein includes, for example, substrates for dying immune tissues such as

True Blu (trade name) commercially available from Kirkegaard Laboratories, Inc. and containing 3,3',5,5'-tetramethylbenzidine and hydrogen peroxide, DAB (3,3'-diaminobenzide) and the like.

The present invention will be further described with reference to working examples thereof. Note, however, that the present invention should not be restricted to these examples.

Example 1:

Preparation of Immobilizing Carrier

50 g of beads of polymethylmethacrylate (PMMA) having an average granule diameter of 7 microns and a density of 1.19 g/cm^3, dyed with a quinophthalone disperse dye, MITSUI ML Colors ML Yellow VF-2 (trade name) commercially available from Mitsui Toatsu Senryo Kabushiki Kaisha, and 5.0 g of particules of calcium phosphate compound having a Ca/P ratio of 1.5, average particle diameter of 2 microns, specific surface area of 12 m^2/g, apparent density of 2.4 g/cm^3 and pore size of 1000 angstroms were blended at 38 to 71℃ for 5 minutes in the Nara Hybridization System NHS-1 (commercially available from Nara Kikai Seisakusho; rated power: 5.5 kw and rated current: 23 A) rotated at 8000 rpm to produce PMMA beads having a coating of calcium phosphate compound applied over a surface of the beads. The resulting coating of calcium phosphate compound had a thickness of

0.27 microns in average. The resulting granular composite had an average granule diameter of 7.5 microns, density of 1.3 g/cm^3 and pore size of 1000 angstroms, and showed a pale yellow color.

Preparation of Testing Element

An antigen for Japanese encephalitis strain Nakayama vaccine was adsorbed on the granular composite obtained in the above step, and centrifuged to remove an excess amount of the antigen. Then, the antigen adsorbed on the composite was immobilized by immersing the composite in a solution of 0.1 % by weight of glutaraldehyde. After immobilization of the antigen, the composite was immersed in a solution of a blocking agent containing casein, trade name "Block Ace" commercially available from Snow Brands Milk Products Co., Ltd. After thorough stirring, the solution was centrifuged to remove an excess amount of the blocking agent. The composite beads having immobilized thereon an antigen for the strain Nakayama vaccine were thus produced.

Evaluation of Testing Element

100 microliters of a phosphate buffer solution (PBS) containing 0.5 w/v % of the composite beads with the immobilized antigen for the strain Nakayama vaccine was added to a series of test tubes. Then, 100 microliters of a rabbit antiserum to said strain Nakayama vaccine was added to each test tube, after said rabbit antiserum was diluted by

doubling to make dilution degrees ranging from 500 times to 32000 times. The mixtures were shaken at room temperature for 45 minutes. After addition of PBS, the mixtures were centrifuged at 1500 rpm for 5 minutes to separate a supernatant to thereby remove any unreacted antibodies.

Thereafter, 500 microliters of 500 times-diluted anti-rabbit IgG peroxidase labelling antibody was added to each of the test tubes and shaken at room temperature for one hour. After centrifugal washing, 50 microliters of a substrate for dying immune tissues, True Blue (trade name) commercially available from Kirkegaard Laboratories, Inc., was added to each test tube, and after one minute, water was added to the test tube to stop the reaction therein. It was found from the observation of the coloring of the beads in the test tubes that a certain level or density of the coloring (bluish green) could be obtained for the dilution degree of the antiserum of from 500 times to 4000 times, and the coloring was weakened with increase of the dilution degree, and that such clear variation of the coloring could be observed until the dilution degree is increased to 16000 times, in comparison with the control containing no antiserum. The above results are evidence that the described detection method enables the visual determination of an amount of the immobilized antigen or antibody on the strain Nakayama vaccine antigen-immobilized

composite beads.

Example 2:

Preparation of Immobilizing Carrier

50 g of nylon beads having an average granule diameter of 5 microns and a density of 1.03 g/cm³, dyed with an anthraquinone disperse dye, MITSUI ML Colors ML Red VF-2 (trade name) commercially available from Mitsui Toatsu Senryo Kabushiki Kaisha, and 7.5 g of particles of hydroxyapatite having a Ca/P ratio of 1.67, average particle diameter of 5 microns, specific surface area of 45 m²/g, apparent density of 1.8 g/cm³ and pore size of 600 angstroms were blended at 32 to 50℃ for 5 minutes in the Nara Hybridization System NHS-1 (commercially available from Nara Kikai Seisakusho; rated power: 5.5 kw and rated current: 23 A) rotated at 8000 rpm to produce nylon beads having a coating of hydroxyapatite applied over a surface of the beads. The resulting coating of hydroxyapatite had a thickness of 0.44 microns in average. The resulting granular composite had an average granule diameter of 5.8 microns, density of 1.13 g/cm³ and pore size of 600 angstroms, and showed a pink color.

Preparation of Testing Element

The procedure of Example 1 was repeated with the proviso that an antigen of A-type influenza virus was adsorbed on the granular composite in place of the antigen

of Japanese encephalitis strain Nakayama vaccine. The composite beads having immobilized thereon an antigen for the influenza virus were thus produced.

Evaluation of Testing Element

50 microliters of a phosphate buffer solution (PBS) containing 0.5 w/v % of the composite beads with the immobilized antigen for the influenza virus was poured into each of 96 wells having a V-shaped bottom in a microplate. Then, 50 microliters of a rabbit antiserum to said influenza virus and of a rabbit antiserum to other viruses were added to the wells of the microplate, after said rabbit antiserums were diluted to make different concentrations. The microplate was shaken at room temperature for one hour. After formation of bead pellets, PBS was added, and the centrifugal washing was repeated twice.

Thereafter, 50 microliters of 500 times-diluted anti-rabbit IgG peroxidase labelling antibody was added to each of the wells. The microplate was shaken at room temperature for one hour, and centrifugally washed with PBS. Then, 25 microliters of a substrate for dying immune tissues, True Blue (trade name) was added to each well, and after one minute, water was added to the well to stop the reaction therein. It was found from the observation of the coloring of the beads in the wells that all the test

elements could not develop a color for the wells containing a rabbit antiserum to the virus other than influenza virus, while the coloring (purple) could be obtained for the wells containing a rabbit antiserum to the influenza virus until the dilution degree of 10000 times. The above results evidence that the described evaluation method enables evaluation with a remarkably high sensitivity.

Example 3:

Preparation of Immobilizing Carrier

50 g of nylon beads having an average granule diameter of 5 microns and a density of 1.02 g/cm^3 and 7.5 g of particules of hydroxyapatite having a Ca/P ratio of 1.67, average particle diameter of 5 microns, specific surface area of 45 m^{2}g, apparent density of 1.8 g/cm^3 and pore size of 600 angstroms were blended at 34 to 47 ℃ for 5 minutes in the Nara Hybridization System NHS-1 (commercially available from Nara Kikai Seisakusho; rated power: 5.5 kw and rated current: 23 A) rotated at 8000 rpm to produce nylon beads having a coating of hydroxyapatite applied over a surface of the beads. The resulting coating of hydroxyapatite had a thickness of 0.45 microns in average. The resulting granular composite had an average granule diameter of 5.8 microns, density of 1.12 g/cm^3 and pore size of 600 angstroms.

Preparation of Testing Element

The procedure of Example 1 was repeated to produce the composite beads having immobilized thereon an antigen for the Japanese encephalitis strain Nakayama vaccine.

Evaluation of Testing Element

100 microliters of a phosphate buffer solution (PBS) containing 0.5 w/v % of the composite beads with the immobilized antigen for the strain Nakayama vaccine was added to a series of test tubes. Then, 100 microliters of a rabbit antiserum to said strain Nakayama vaccine was added to each test tube, after said rabbit antiserum was diluted by doubling to make dilution degrees ranging from 500 times to 32000 times. The test tubes were shaken at room temperature for 45 minutes. After addition of PBS, the mixtures were centrifuged at 1500 rpm for 5 minutes to separate a supernatant to thereby remove an unreacted antibody.

Thereafter, 500 microliters of 500 times-diluted anti-rabbit IgG peroxidase labelling antibody was added to each of the test tubes and shaken at room temperature for one hour. After centrifugal washing, 50 microliters of a substrate for dying immune tissues, True Blue (trade name) was added to each test tube, and after one minute, water was added to the test tube to stop the reaction therein. It was found from the observation of the coloring of the beads in the test tube that a certain level or density of the coloring (blue) could be obtained for the dilution degree

of the antiserum of from 500 times to 4000 times, and the coloring was weakened with increase of the dilution degree, and that such clear variation of the coloring could be observed until the dilution degree is increased to 16000 times, in comparison with the control containing no antiserum. The above results are evidence that the described detection method enables the visual determination of an amount of the immobilized antigen or antibody on the strain Nakayama vaccine antigen-immobilized composite beads.

Example 4:

Preparation of Testing Element

The procedure of Example 1 was repeated with the proviso that the porous granules of hydroxyapatite having a grain diameter of 300 to 600 microns (average grain diameter of 450 microns), pore size of 0.005 to 0.01 microns and specific surface area of 10 m^2/g were used as the immobilizing carrier in place of the granular colored composite having an average granule diameter of 7.5 microns, density of 1.3 g/cm^3 and pore size of 1000 angstroms. The hydroxyapatite beads having immobilized thereon an antigen for the Japanese encephalitis strain Nakayama vaccine were thus produced.

Evaluation of Testing Element

100 microliters of a phosphate buffer solution (PBS) containing 0.5 w/v % of the hydroxyapatite beads with the

immobilized antigen for the strain Nakayama vaccine was added to a series of test tubes. Then, 100 microliters of a rabbit antiserum to said strain Nakayama vaccine was added to each test tube, after said rabbit antiserum was diluted by doubling to make dilution degrees ranging from 500 times to 32000 times. The test tubes were shaken at room temperature for 45 minutes. After addition of PBS, the mixtures were centrifuged at 1500 rpm for 3 minutes to separate a supernatant to thereby remove an unreacted antibody.

Thereafter, 500 microliters of 500 times-diluted anti-rabbit IgG peroxidase labelling antibody was added to each of the test tubes and shaken at room temperature for one hour. After centrifugal washing, 50 microliters of a substrate for dying immune tissues, True Blue (trade name) was added to each test tube, and after one minute, water was added to the test tube to stop the reaction therein. It was found from the observation of the coloring of the beads in the test tubes that a certain level or density of the coloring (blue) could be obtained for the dilution degree of the antiserum of from 500 times to 4000 times, and the coloring was weakened with increase of the dilution degree, and that such clear variation of the coloring could be observed until the dilution degree is increased to 16000 times. The above results are evidence that the described detection method enables the visual determination of an

amount of the immobilized antigen or antibody on the strain Nakayama vaccine antigen-immobilized hydroxyapatite beads.

Example 5:

Preparation of Immobilizing Carrier

Porous granules of hydroxyapatite having an average grain diameter of 3.5 microns and a Ca/P ratio of 1.67 were applied to a nonwoven fabric having a thickness of 0.2 mm and a size of 5 mm × 10 mm consisting of 50 % by weight of polyethylene and 50 % by weight of polyethylene terephthalate ester, followed by thermal treatment to produce a ceramics-carried fibrous composite having substantially uniformly carried thereon 24 % by weight of the hydroxyapatite granules.

Preparation of Testing Element

A 256 HA (hemagglutination) value of A-type influenza virus was adsorpted onto the fibrous composite, and then immobilized on the composite by immersing the composite in a solution of 0.05 % by weight of glutaraldehyde. After immobilization of the influenza virus, the fibrous composite was immersed in a four times-diluted solution of a blocking agent containing casein, Block Ace (trade name), to selectively mask the influenza virus-unadsorbed sites of the composite. The fibrous composite was again treated with a solution of 0.05 % by weight of glutaraldehydethe to immobilize said

blocking agent. Further, the fibrous composite was immersed in a neutral buffer solution containing the blocking agent to inactivate an aldehyde residue remaining on the composite as a function of the chemical bonding between the aldehyde residue and proteins. The influenza virus-bonded fibrous composite as the testing element was thus produced.

Evaluation of Testing Element

The resulting influenza virus-bonded fibrous composite was added to test tubes each of which contained a diluted rabbit antiserum to said influenza virus or a diluted rabbit antiserum to other viruses in different concentrations. The mixture in the test tubes were shaken at room temperature for one hour. After washing with PBS, 500 microliters of 500 times-diluted anti-rabbit IgG peroxidase labelling antibody was added to each of the test tubes, and shaken at room temperature for one hour.After centrifugal washing with PBS, 200 microliters of a substrate for dying immune tissues, True Blue (trade name), was added to each test tube, and after one minute, water was added to the test tube to stop the reaction therein. It was found from the observation of the coloring of the fibrous composite in the test tube that all the test elements could not develop a color for the test tubes containing a rabbit antiserum to the virus other than the

influenza virus, while the coloring (purple) could be obtained for the test tubes containing a rabit antiserum to the influenza virus until the dilution degree of 10000 times. The above results are evidence that the described evaluation method enables the evaluation with a remarkably high sensitivity.

CLAIMS

1. A method of evaluating a testing element having an immobilized antigen or antibody on an immobilizing carrier thereof, the method comprising the steps of:-

contacting the testing element with a solution containing an antibody or antigen reactive with said immobilized antigen or antibody,

specifically bonding a labelling compound to said reactive antibody or antigen to thereby cause a color developing reaction of said labelling compound, and

determining an amount of the antigen or antibody immobilized on said testing element as a function of the level of the produced color.

2. An evaluating method according to claim 1 in which said immobilizing carrier comprises particles of a calcium phosphate compound having a Ca/P ratio of about 1.0 to 2.0 and an average particle diameter of about 1 to 10000 microns.

3. An evaluating method according to claim 1 in which said immobilizing carrier comprises a fibrous aggregate with the carried particles of a calcium phosphate compound having an average particle diameter of about 0.01 to 200 microns and a Ca/P ratio of about 1.0 to 2.0.

4. An evaluating method according to claim 1 in which

said immobilizing carrier comprises a granular composite of polymer comprising polymeric granules having coated on a surface thereof a calcium phosphate compound having a Ca/P ratio of about 1.0 to 2.0, at least a part of particles of said calcium phosphate compound being penetrated in said polymeric granules.

5. An evaluating method according to claim 1 in which said immobilizing carrier comprises a granular composite of polymer comprising polymeric granules having coated on a surface thereof a calcium phosphate compound having a Ca/P ratio of about 1.0 to 2.0, said polymeric granules or said granular composite being dyed.

6. An evaluating method according to any one of claims 1 to 5 in which said labelling compound is an antigen or antibody which contains at least one of peroxidase, glucose oxidase, tyrosinase, acidic phosphatase or alkaline phosphatase as a labelling enzyme.

7. An evaluating method according to any one of claims 1 to 6 in which said color-developing reaction is made based on use of a substrate capable of providing color as a result of its reaction with said labelling compound.

8. An evaluating method according to any preceding claim in which said testing element is contacted with a solution containing a known amount of antibody or antigen reactive with said immobilized antigen or antibody.

9. A method of evaluating a testing element having an immobilized antigen or antibody on an immobilizing carrier thereof, substantially as herein described.